Takashi MATSUSHIMA*: Study on the mode of action of filipin, a polyene type antifungal antibiotic

松島崇*: ポリエン型抗カビ性物質フィリィピンの作用機構について

Introduction

Filipin is a potent antifungal antibiotic produced by *Streptomyces filipinensis* (1). It has an empirical formula $C_{30}H_{50}O_{10}$ and belong to the conjugated polyene type. In common with other polyenes, filipin is susceptible to autoxidation especially in the presence of light and the oxidized filipin loose the antifungal activity (2).

Although filipin will not be used practically because of this untable character, the mode of action of this antibiotic is of academic interest. Gottlieb et al (3) found that cholesterol and some other sterols could reverse filipin inhibition of certain fungi. The author found this to be true in *Rhizopus nigricans* and some new findings were added and reported herein.

Materials and Methods

Rhizopus nigricans Ehrenberg, a phycomycete, was chosen as a test fungus because of its rapid growth and high sensitivity to filipin. The basic medium composed, per liter basis, of glucose, 10 g; L glutamic acid, 2.1 g; K_2HPO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; Fe^{+++}, 0.2 mg; Zn^{++}, 0.2 mg; Mn^{++}, 0.1 mg; and agar, 15 g. All test chemicals were added to the autoclaved basic medium and pH of the media was adjusted to 6.5 after adding test chemicals. The sporangiospore suspension was streaked over the Petri plates aud the inoculated plates were incubated in darkness at 25°C. for 72 hr. The growth of the fungus was recorded with arbitrary measures of −, −, ++, and +++. All organic chemicals used were the product of the Nutritional Biochemicals Corp., except otherwise stated.

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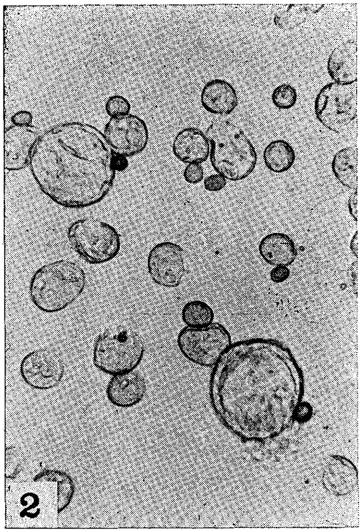
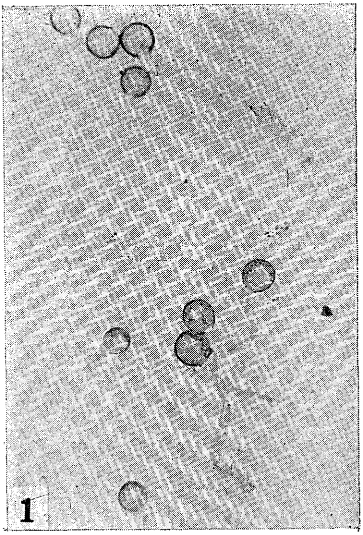
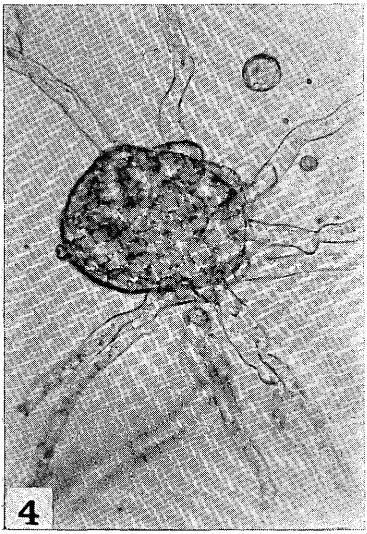
Results

I. Abnormal development of the sporangiospores in the presence of filipin.







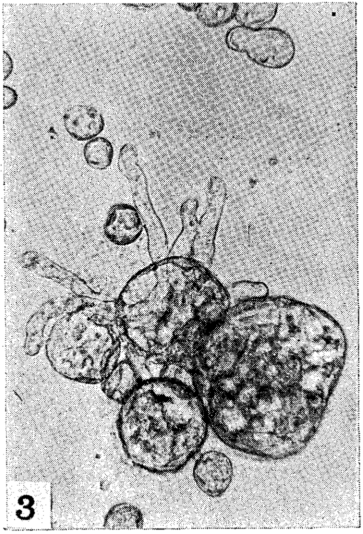


Fig. 1. Normal germination of the spores, ×400.
Fig. 2. Abnormal enlargement of the spores, ×400.
Fig. 3-4. Germination of the enlarged spores, ×400.

The complete inhibition of this fungus was attained with 8 μg/ml filipin on the basic medium. It was found that the sporangiorpores swelled enormously on the basic medium in the presence of subcritical concentrations of filipin, which is

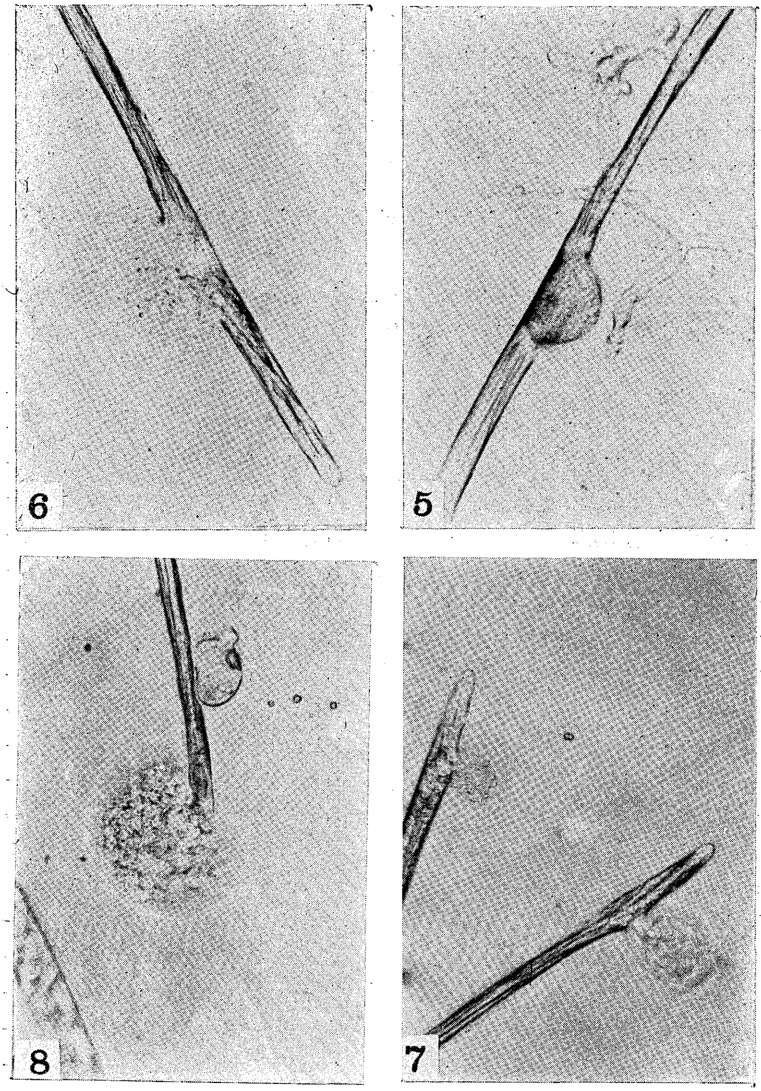


Fig. 5-8. Swelling, rupture, and oozing-out of the protoplasm from the hyphae, ×800.

5–6 μg/ml. The diameter of the enlarged spores attained up to 80 μ compared to 13 μ of the germinating spores on the basic medium (Fig. 1–4). It was seen that only enlarged spores could germinate and produce "octopus" — like bodies (Fig. 3–4). Frequent ruptures of the hyphae from these enlarged spores and subsequent oozing-out of the protoplasm could be observed (Fig. 5–8). Some study has been made concerning chemical compounds which produce a similar abnormal phenomenon and it was found that Na-dodecylsulfate, a surface active agent, produce a similar enlargement of the spores.

II. Reversing ability of the following chemicals for the inhibition caused by 8 μg/ml filipin.

The concentrations employed were 1mM of the basic medium, except otherwise stated. (+and−denote "positive" and "negative" respectively.)

Casein hydrolyzate (enzymatic) (Difco), 2 g/l	+
Yeast extract (Difco), 2 g/l	+
Malt extract (difco), 2 g/l	−
Peptone (Difco), 2 g/l	−
Vitamin mixture (thiamine hydrochloride, 1 mg; pyridoxine hydrochloride, 1 mg; biotin, 50 μg; inositol, 50 mg/l.)	−
L alpha alanine	−
beta alanine	−
amino acetic acid	−
L arginine	−
L asparagine	−
L aspartic acid	−
DL citrulline	−
L cysteine	+
L cystine	+
DL dihydroxyphenylalanine	−
L glutamic acid	−
L glutamine	−
L histidine	−
L isoleucine	−
L leucine	−
L lysine	−
L methionine	−
L ornithine	−
L phenylalanine	−
L proline	−
DL serine	−
Dl threonine	−
L tyrosine	−
L valine	−
glutathione (reduced form)	+
L glutamic acid plus aminoacetic acid	−
glucose-1-phosphate	−
fructose-6-phosphate	−
fructose-1, 6-diphosphate	−
nucleic acid (yeast)	−
ribose nucleic acid	−

adenosine	—	cholesterol (Merck)	+
uridine	—	steraric acid (Merck)	+
cytidine	—	linoleic acid	—
alpha ketoglutaric acid	—	linolenic acid	—
L ascorbic acid	—	beta-carotene, crystalline	—
hydroquinone (Merck)	—		

Results of further experiments with the substances which were found to be effective.

Filipin conc. in μg/ml / Antagonists conc. in mM	4	8	16	32
Control	⧻	—	—	—
Cholesterol $\frac{1}{1280}$	⧻	—	—	—
$\frac{1}{640}$	⧻	+	—	—
$\frac{1}{320}$	⧻	⧻	—	—
$\frac{1}{160}$	⧻	⧻	—	—
$\frac{1}{80}$	⧻	⧻	⧻	—
1	⧻	⧻	⧻	⧻
Stearic acid $\frac{1}{4}$	⧻	⧺	—	—
$\frac{1}{2}$	⧻	⧺	+	—
1	⧻	⧺	+	—

Filipin conc. in μg/ml / Antagonists conc. in mM	4	8	16	32
Glutathione $\frac{1}{8}$	⧻	⧺	—	—
$\frac{1}{4}$	⧻	⧻	⧺	—
$\frac{1}{2}$	⧻	⧻	⧺	+
1	⧻	⧻	⧻	⧺
Cysteine $\frac{1}{4}$	⧻	—	—	—
$\frac{1}{2}$	⧻	⧺	—	—
1	⧻	⧻	⧺	—
2	⧻	⧻	⧺	+

Discussion

Gottlieb et al (3) found that cholesterol and certain other sterols are active in offsetting filipin inhibition of *Penicillium oxalicum*, *Aspergillus niger* and *Hansenula subpelliculosa* in very low concentrations. They proposed a hypothesis that these sterols are probobly essential metabolites and filipin will either prevent the synthesis of the sterols or competitively replace them.

III. Synergistic effect of sodium chloride to filipin inhibition.

NaCl conc. in w/v% Filipin conc. in μg/ml	0	2	4	8	12
0	+++	++	++	+	−
2	+++	+	−	−	−
4	+++	−	−	−	−
8	−	−	−	−	−

In *Rhizopus nigricans*, cholesterol was found to be very effective in reversing filipin inhibition. Steraric acid is moderately effective but linoleic acid, linolenic acid, and beta-carotene had no reversing ability.

Glutathione in reduced form and cysteine were discovered to have reversing power in competitive manner. Since both glutathione and cysteine, and filipin are quite easily oxidizable compounds, the reversing effect will not be a direct chemical reaction between them.

The marked synergistic effect of NaCl on filipin inhibition indicates that the primary site of action of this antibiotic will be on the cell membrane regulating the osmotic barriers. This hypothesis could be backed up by the abnormal development of the spores in the presence of filipin.

Further study is required to clarify the mode of action of this antibiotic and the mechanism of the reversal, especially in connection with the physiology of the cell membrane.

Summary

Complete inhibition of the development of *Rhizopus nigricans* was attained by 8 ppm fillipin. With subcritical concentrations of this antibiotic, the abnormal enlargement of the sporangiospores and the rupture of the germ tubes were consistently observed. The inhibition was reversed very effectively by cholesterol, and less effectively by glutathione, cysteine, and steraric acid. NaCl had a marked synergistic effect for filipin inhibition.

Cell membrane was suggested as the probable site of action of this antibiotic.

Acknowledgment

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○高等植物分布資料 (11) Materials for the distribution of vascular plants in Japan

○スキヤクジャク *Adiantum diaphanum* Bl.

熱帯シダのスキヤクジャクが壱岐に産することは外山三郎氏によって報告（本誌 **29**: 326) されたが，筆者はその後さらに大小二つの群をこの島で発見した。また同教授から，平戸の大島にもあるらしいとのことを聞いていたが，機会あって去る 5 月 21 日踏査しその存在を確認することができた。そこでこれらについて概略を報告する。

壱岐の大きい方の群落は，既報の群落から数 m 離れた所にある掘り割られた水路の両岸である。それは水面から 40〜120cm の高さに密生し，北岸では 15•5m，南岸では 17•5m にわたり帯のように長く続いて実に美事である。小さい方の群落はここから 100 m ばかりの所にある人家の裏(北側)の崖である。横 3 m，高さ 0〜50cm の間に雑草を混えず密生している。さらに小さい群落は附近に数個ある。

平戸の大島は平戸島の北 7 km にある小さい島で，大島村という一村をなしている。群生地は海岸から約 500 m はいった所の人家の裏の崖で，その環境は壱岐島のものに酷似している。横 9m，高さ 30〜50 cm の間にまばらに，あるいは密に群生している。中央は崖の上から流れ落ちる水のため 2m あまり切れている。(品川鉄摩)